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Reduced Live Organism Recovery and Lack of Hydrosalpinx in Mice Infected with Plasmid-Free *Chlamydia muridarum*

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Plasmid-free *Chlamydia trachomatis* and *Chlamydia muridarum* fail to induce severe pathology. To evaluate whether the attenuated pathogenicity is due to insufficient infection or inability of the plasmidless chlamydial organisms to trigger pathological responses, we compared plasmid-competent and plasmid-free *C. muridarum* infections in 5 different strains of mice. All 5 strains developed hydrosalpinx following intravaginal inoculation with plasmid-competent, but not inoculation with plasmid-free, *C. muridarum*. The lack of hydrosalpinx induction by plasmid-free *C. muridarum* correlated with significantly reduced live organism recovery from the lower genital tract and shortened infection in the upper genital tract. The plasmid-free *C. muridarum* organisms failed to induce hydrosalpinx even when the organisms were directly inoculated into the oviduct via an intrabursal injection, which was accompanied by significantly reduced survival of the plasmidless organisms in the genital tracts. Furthermore, plasmid-competent *C. muridarum* organisms after UV inactivation were no longer able to induce hydrosalpinx even when directly delivered into the oviduct at a high dose. Together, these observations suggest that decreased survival of and shortened infection with plasmid-free *C. muridarum* may contribute significantly to its attenuated pathogenicity. We conclude that adequate live chlamydial infection in the oviduct may be necessary to induce hydrosalpinx.

Sexually transmitted infection with *Chlamydia trachomatis* can cause pathology in the upper genital tract, including hydrosalpinx, a laparoscope-detectable marker of tubal factor infertility (1). Although inflammatory responses induced by persistent chlamydial organisms have been hypothesized to contribute significantly to upper genital tract pathology (2, 3), it remains unknown whether live organism infection in the fallopian tube is necessary for induction of hydrosalpinx and how chlamydial organisms spread to the fallopian tube and trigger hydrosalpinx-causing inflammation. It has been difficult to directly address these questions in humans. The species *Chlamydia muridarum*, although causing no known human diseases, has been extensively used to study the pathogenic mechanisms and immune responses of *C. trachomatis* (4, 5). Genital tract infection of mice with *C. muridarum* can cause hydrosalpinx that closely mimics the tubal pathology induced by *C. trachomatis* in humans.

When intravaginal infections with *C. muridarum* in C57BL/6J and C3H/HeN mice were compared, Shah et al. found that C3H/HeN mice developed more robust pyosalpinx (acute inflammatory infiltration in the lumen of the oviduct) on day 28 and more severe hydrosalpinx (fibrotic occlusion) on day 56 after infection. This observation led the authors to correlate acute inflammatory responses with the development of hydrosalpinx (6). However, it is still unclear whether live organism infection in the oviduct is necessary for the induction of hydrosalpinx, since live organism shedding was monitored only in the lower, but not the upper, genital tract (6). Darville et al. identified a role of Toll-like receptor 2 (TLR2)-mediated signaling pathways in *C. muridarum*-induced upper genital tract pathology examined microscopically on day 35 after infection (7). However, it is unknown whether the TLR2-mediated signaling pathway alone is sufficient for *C. muridarum* induction of long-lasting hydrosalpinx, since TLR2-null ($TLR2^{-/-}$) mice developed chronic inflammatory pathology in the oviduct as severe as that of their heterozygous ($TLR2^{+/-}$) littermates, with a median oviduct dilation score of ~2 for $TLR2^{-/-}$ and ~3 for $TLR2^{+/-}$ mice despite the significantly reduced inflammatory scores in the mesosalpingeal tissues of the $TLR2^{-/-}$ mice (7). More importantly, many questions remain unanswered regarding the mechanism, location, duration, and extent of inflammatory signaling pathways activated during chlamydial infection. Our hypothesis is that live organism infection in oviduct epithelial cells may be necessary to induce hydrosalpinx, which is consistent with the observation that epithelial cells actively infected with chlamydial organisms are more inflammatory than cells stimulated with noninfectious chlamydial antigens (2, 8, 9).

The observation that plasmid-free *C. trachomatis* or *C. muridarum* organisms were highly attenuated in primate ocular (10) or mouse genital tract (11) tissues suggests a critical role of the chlamydial plasmid in chlamydial pathogenesis. The chlamydial plasmid includes 8 putative open reading frames (ORFs) and also regulates the expression of more than 20 other genes, including *glgA*, at the transcription level (12). Intravaginal infection with plasmid-free *C. muridarum* did not activate the TLR2 signaling pathway and failed to induce hydrosalpinx (11). However, it is not clear whether the lack of TLR2 signaling during plasmid-free *C. muridarum* infection was due to insufficient infection or lack of ligands (or virulence factors) required for activating TLR2 signaling. We hypothesize that inadequate infection in the oviduct by

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plasmid-free *C. muridarum* may contribute significantly to the attenuated-pathology phenotype.

To test the above hypotheses, we compared plasmid-competent and plasmid-free *C. muridarum* infections in 5 different strains of mice in the current study. Intravaginal inoculation with plasmid-competent, but not plasmid-free, *C. muridarum* induced significant hydrosalpinx in all 5 strains. The lack of hydrosalpinx in plasmid-free *C. muridarum*-infected mice was accompanied by both decreased levels of live organism recovery from the lower genital tract and shortened infection in the upper genital tract. The plasmid-free *C. muridarum* organisms were less able to survive in the upper genital tract, since the ratios of genome copies versus numbers of live organisms recovered from the oviduct were always higher for *C. muridarum* without a plasmid than for those with a plasmid. When *C. muridarum* organisms were directly inoculated into the oviduct, plasmid-free *C. muridarum* did not maintain a robust infection in the genital tract and failed to induce hydrosalpinx, while plasmid-competent *C. muridarum* did both. The plasmid-competent *C. muridarum* organisms were no longer able to induce pathology after UV inactivation. Thus, the persistence of high levels of live chlamydial organisms in the oviduct may be necessary for the induction of hydrosalpinx.

MATERIALS AND METHODS

Chlamydial organisms and infection. The plasmid-competent *C. muridarum* strain (Nigg) used in the current study was propagated in HeLa cells (human cervical carcinoma epithelial cells; ATCC CCL2.1), purified, aliquoted, and stored as described previously (13). The plasmid-free *C. muridarum* strain, designated CMUT3, was selected from plasmid-competent *C. muridarum* using novobiocin plus a plaque assay, as described previously (11, 14–16). In addition to validating the lack of a plasmid in CMUT3, we sequenced its entire genome. At the same time, for comparison purposes, we also sequenced the genome of a previously published plasmid-free clone, CM972 (14). These two genome sequences are nearly identical. Both genomes contain a frameshift mutation in the gene TC0412 (a homolog of CT135). However, the precise locations of nucleotide insertion are different, with a T insertion at nucleotide 473154 (codon 133), resulting in premature termination at codon 146 of CM972 TC0412, and a T insertion at nucleotide 472839 (codon 28), resulting in premature termination at codon 47 of CMUT3 TC0412. The distinct insertion mutation patterns indicated that CM972 and CMUT3 were derived from different sources.

Female BALB/cJ (stock number 000651), C57BL/6J (000664), SJL/J (000686), C3H/HeJ (000659), and CBA/J (000656) mice were purchased at the age of 5 to 6 weeks from Jackson Laboratories (Bar Harbor, ME). A mouse use protocol was approved by the Committee on the Ethics of Laboratory Animal Experiments of the University of Texas. Each mouse was inoculated intravaginally with 2×10^5 inclusion-forming units (IFU) of live *C. muridarum* plasmid-competent Nigg or plasmid-free CMUT3 organisms in 20 μl of SPG (sucrose-phosphate-glutamate buffer) or intrabursally with different amounts of organisms, as indicated, in 10 μl of SPG. For some experiments, plasmid-competent *C. muridarum* organisms were UV inactivated as described previously (17, 18) before inoculation. Five days prior to infection, each mouse was injected subcutaneously with 2.5 mg Depo-Provera (Pharmacia Upjohn, Kalamazoo, MI) to synchronize the estrus cycle and increase mouse susceptibility to chlamydial infection. The intrabursal injection was carried out as described previously (19). For *in vitro* infection, HeLa cells grown on coverslips in 24-well plates containing Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD) with 10% fetal calf serum (FCS) (Gibco BRL) at 37°C in an incubator supplied with 5% CO_2 were inoculated with *C. muridarum* as described previously (3). The infected cultures were processed for immunofluorescence assay as described below.

Monitoring live organism recovery from vaginal swab and genital tract tissues. To monitor live organism shedding, vaginal swabs were taken on different days after intravaginal or intrabursal infection. Each swab was dissolved in 500 μl of ice-cold SPG, followed by vortexing with glass beads, and the released organisms were titrated on HeLa cell monolayers in duplicate as described previously (20). To monitor upper genital tract infection, the genital tract tissue was harvested sterilely from each mouse on different days after infection, as indicated for individual experiments. Each tissue was cut into different segments, as indicated for individual experiments. In some cases, the genital tract was divided into 3 portions, the vagina/cervix, uterus/uterine horn (both sides from the same mouse were put together as a single segment), and oviducts/ovaries (both sides from the same mouse were pooled as a single tissue sample), while in others, the left and right uterine horns and oviduct/ovaries were separated. Each tissue segment sample was homogenized in 300 μl of SPG using a 2-ml mini-tissue grinder (Fisher Scientific, Pittsburgh, PA). After brief sonication, the released live organisms were titrated in HeLa cells as described above. The total number of IFU per swab or tissue segment was calculated based on the number of IFU per field, the number of fields per coverslip, dilution factors, and inoculation and total sample volumes. An average was taken from the serially diluted and duplicate samples for any given swab/tissue. The calculated total number of IFU per swab or tissue was converted into $\log_{10}$, and the $\log_{10}$ IFU was used to calculate the mean and standard deviation for each group at each time point.

Quantitating *C. muridarum* genome copies in genital tract tissues using qPCR. A portion of each sample (50 μl out of 300 μl) was subjected to DNA extraction with a QIAamp DNA Minikit-250 (Qiagen, Frederick, MD) following the manufacturer's instructions. Each DNA preparation was resuspended in 100 μl of water, and 1 μl of the DNA sample was aliquoted for quantitative PCRs (qPCRs) using the primers complementary to the chlamydial 16S rRNA coding region, including a forward primer, 5′-CGCCTGAGGAGTACACTCGC-3′, and a reverse primer, 5′-CCAACACCTCACGGCACGAG-3′, for amplifying a 208-bp fragment and a probe primer, 5′-CACAAGCAGTGGAGCATGTGGTTTAA-3′; all primers were synthesized by Integrated DNA Technologies (Coralville, IA) for real-time detection. A plasmid standard containing the 208-bp fragment of the chlamydial 16S ribosomal gene was used for qPCR quantification. The PCR was carried out in a total volume of 20 μl in a CFX96 Touch Deep Well Real-Time PCR Detection System with iQ Supermix real-time PCR reagent (Bio-Rad, Hercules, CA). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. The results were expressed as the total number of genome copies per sample and plotted in $\log_{10}$.

Evaluating mouse genital tract tissue pathologies and histological scoring. Mice were sacrificed on different days postinfection, as indicated for individual experiments, and urogenital tract tissues were isolated. Before the tissues were removed, *in situ* gross examination was performed for evidence of oviduct hydrosalpinx or any other related abnormalities of oviducts. Mice with hydrosalpinx on either side of the oviducts were determined to be hydrosalpinx positive when calculating the percentage of mice positive for hydrosalpinx. The severity of hydrosalpinx was scored based on the following criteria: 0, no hydrosalpinx; 1, hydrosalpinx detectable only under stereoscope or microscope examination; 2, hydrosalpinx clearly visible with the naked eye but smaller than the ovary on the same side; 3, hydrosalpinx size equal to that of the ovary on the same side; or 4, hydrosalpinx size larger than that of the ovary on the same side. Scores from both sides of the oviducts from the same mouse were combined as the score for that mouse and used for calculating means and standard deviations for each group of animals. The excised tissues, after photographing, were fixed in 10% neutral formalin, embedded in paraffin, and serially sectioned longitudinally (5 μm/section). Efforts were made to include the cervix, both uterine horns, and the oviducts, as well as lumenal structures of each tissue in each section. The sections were stained with hematoxylin and eosin (H&E) as described previously (18,

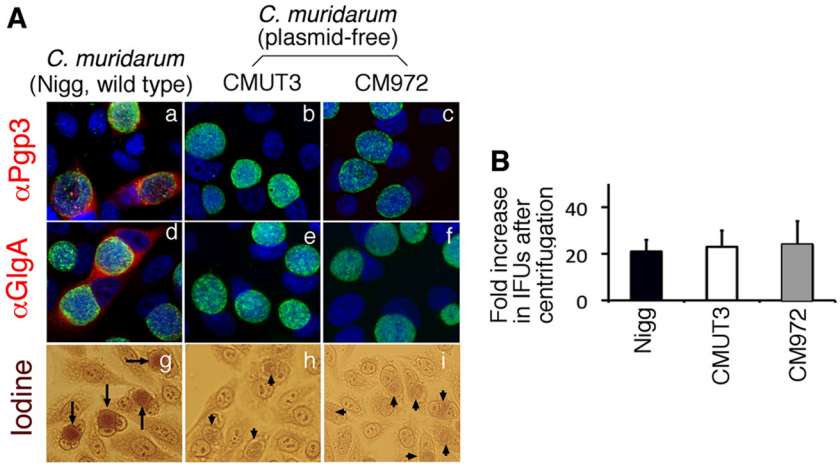

FIG 1 Comparison of plasmid-free *C. muridarum* CMUT3 and CM972. (A) Plasmid-competent Nigg (wild type) (images a, d, and g) and plasmid-free CMUT3 (b, e, and h) and CM972 (c, f, and i) *C. muridarum* organisms were used to infect HeLa cells, and the infected cultures were processed for immunofluorescence labeling with a rabbit anti-*C. muridarum* antibody (green) (a to f) and mouse anti-Pgp3 (αPgp3) (a to c) or anti-GlgA (αGlgA) (d to f) antibodies (red) plus DNA dye (blue) (a to f) or iodine staining (brown) (g to i). Note that CMUT3 and CM972 were unable to express detectable levels of either Pgp3 or GlgA or to accumulate glycogen detectable with iodine, while the wild-type Nigg organisms were able to do all these things. Inclusions stained positive for glycogen are marked with arrows (g), while negative inclusions are marked with arrowheads (h and i). (B) The ratios of yields of live organisms recovered from HeLa cells infected with Nigg, CMUT3, or CM972 with or without centrifugation were compared. Centrifugation enhanced infectivity for all *C. muridarum* organisms by >20-fold regardless of the plasmid status. The error bars indicate standard deviations (SD).

21, 22). The H&E-stained sections were assessed by a pathologist blinded to mouse treatment and scored for severity of inflammation and lumenal dilation as described previously (21, 22). The uterine horns and oviducts were scored separately (only the oviduct scores were used in the current study). Scoring for dilation of the uterine horn or oviduct was as follows: 0, no significant dilation; 1, mild dilation of a single cross section; 2, one to three dilated cross sections; 3, more than three dilated cross sections; and 4, confluent pronounced dilation. Scoring for chronic inflammatory cell infiltrates (at the chronic stage of infection, the infiltrates mainly contain mononuclear cells, while at the acute stage, neutrophils dominate the infiltration) was as follows: 0, no significant infiltration; 1, infiltration at a single focus; 2, infiltration at two to four foci; 3, infiltration at more than four foci; and 4, confluent infiltration. Scores assigned to individual mice were calculated as means and standard errors per group for statistical analyses. Individual scores and medians were plotted.

Immunofluorescence assay. HeLa cells grown on coverslips with chlamydial infection were fixed and permeabilized for immunostaining as described previously (23, 24). Hoechst dye (blue; Sigma) was used to visualize nuclear DNA. For titrating IFU from swab and tissue homogenate samples, a mouse anti-chlamydial lipopolysaccharide (LPS) antibody (clone MB5H9 [unpublished observation]) plus a goat anti-mouse IgG conjugated with Cy3 (red; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to visualize chlamydial inclusions. Hoechst dye (Sigma-Aldrich, St. Louis, MO) was used to label DNA (blue). All immunofluorescence-labeled samples were observed under an Olympus AX-70 fluorescence microscope equipped with multiple filter sets (Olympus, Melville, NY).

Statistical analyses. The Kruskal-Wallis test was used to analyze the differences in IFU and genome copies recovered from mouse swabs and tissues. The semiquantitative pathology scores were analyzed with the Wilcoxon rank sum test. Fisher's exact test was used to analyze category data, including the percentage of mice with hydrosalpinx.

Nucleotide sequence accession numbers. The consensus genome sequence for CMUT3 is available under GenBank accession number CP006974, while the genome sequence for CM972 (14) can be accessed under accession number CP006975.

RESULTS

Induction of hydrosalpinx in 5 strains of mice by intravaginal infection with plasmid-competent but not plasmid-free *C. muridarum*. To investigate the mechanisms of chlamydial plasmid-dependent pathogenicity, we isolated a plasmid-free *C. muridarum* strain designated CMUT3 and, further, compared CMUT3 with a previously published plasmid-free *C. muridarum* clone, CM972 (14). As shown in Fig. 1, both CMUT3 and CM972 failed to express detectable levels of either Pgp3 or GlgA and to accumulate glycogen detectable with iodine, while the wild-type Nigg organisms were able to do all these things. Centrifugation enhanced infectivity for all *C. muridarum* organisms by >20-fold regardless of the plasmid status. The above-mentioned results suggested that the two plasmid-deficient *C. muridarum* clones behaved very similarly, which is consistent with their similarity at the genetic level. Both CMUT3 and CM972 lacked plasmids and had almost identical genomes. Both genomes contained a frameshift mutation in TC0412 (a homolog of CT135 [25]). However, the precise locations of the nucleotide insertions are different, with a T insertion at nucleotide 472839 (codon 28), resulting in premature termination at codon 47 of CMUT3 TC0412, and a T insertion at nucleotide 473154 (codon 133), resulting in premature termination at codon 146 of CM972 TC0412 (26). Although the precise insertion mutation patterns were different in CMUT3 and CM972, the result of both mutations was the disruption of TC0412.

We then compared the *in vivo* pathogenicity of CMUT3 with that of the wild-type *C. muridarum* Nigg from which CMUT3 was derived. As shown in Fig. 2, five different strains of mice—BALB/cJ (H-2^d haplotype), SJL/J (H-2^s), C57BL/6J (H-2^b), C3H/HeJ (H-2^k), and CBA/J (H-2^a)—each with a unique H-2 haplotype and varying backgrounds, developed significant hydrosalpinx, with incidences ranging from 71% in BALB/cJ to 100% in SJL/J. The hydrosalpinx mean severity scores were from 3.58 in C3H/HeJ to 7.1 in SJL/J (the maximal score for a given mouse is 8).

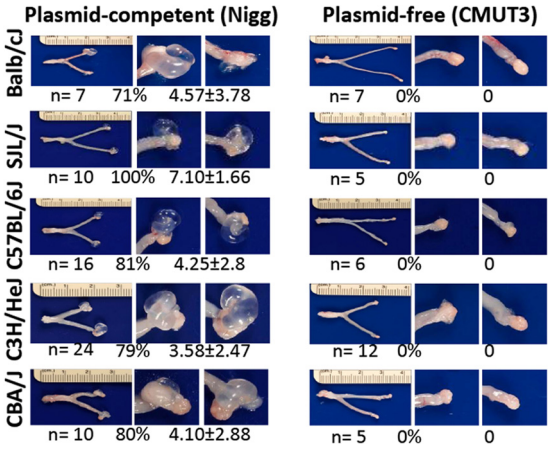

FIG 2 Lack of hydrosalpinx in 5 different strains of mice following intravaginal infection with plasmid-free *C. muridarum*. Five strains of mice, BALB/cJ, SJL/J, C57BL/6J, C3H/HeJ, and CBA/J, as indicated on the left, were infected with either plasmid-competent Nigg or plasmid-free CMUT3 *C. muridarum*. Sixty days after infection, the mice were sacrificed to observe the gross appearance of the upper genital tract. One representative image of the entire genital tract from each strain of mice is presented, with the vagina/cervix at the left and the oviduct/ovary at the right. In the middle and right images, the oviduct/ovary portion is magnified for clearer identification and scoring of hydrosalpinx. The total number of mice and the incidence (percent) and severity ($x \pm$ SD) of hydrosalpinx in each group are shown under the representative image. Note that although Nigg induced a high incidence of severe hydrosalpinx in all 5 strains of mice, CMUT3 failed to do so.

The gross pathology of hydrosalpinx was also validated at a microscopic level (Fig. 3), showing that the wild-type Nigg, but not the plasmid-free CMUT3, induced significant inflammatory infiltration and oviduct lumenal dilation. It is well known that *C.*

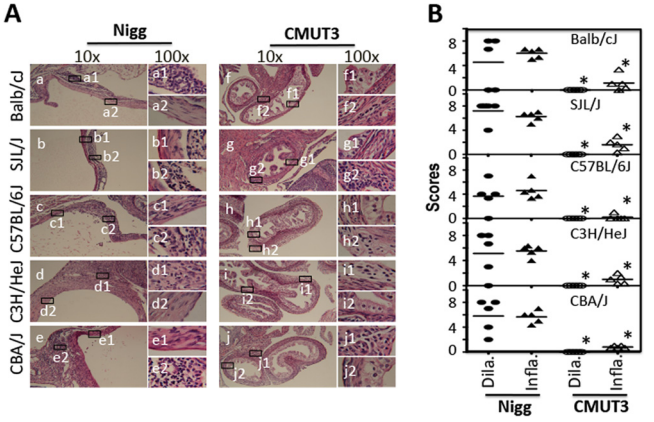

FIG 3 Lack of significant oviduct histopathology following intravaginal infection with plasmid-free *C. muridarum*. Sections from mouse oviduct tissues shown in Fig. 2 were made for H&E staining (only 5 mice from each strain were randomly selected for oviduct histopathology evaluation). (A) Representative images from each of the 5 mouse strains taken under both low-power (10× objective lens) (left images) and high-power (100× objective lens) (right images) magnification. The areas where high-magnification images were taken are boxed in the 10× images. Note that oviducts from all mice infected with *C. muridarum* Nigg, but not CMUT3, displayed severe lumenal dilation and chronic inflammatory infiltration. (B) The severity of both lumenal dilation (Dila.) (solid and open circles) and the inflammatory infiltration (Infla.) (solid and open triangles) was semiquantitatively scored as described in Materials and Methods. The scores for individual mice and the medians for individual mouse strains (horizontal lines) are shown on the *y* axis. *, $P < 0.05$ (Wilcoxon rank sum).

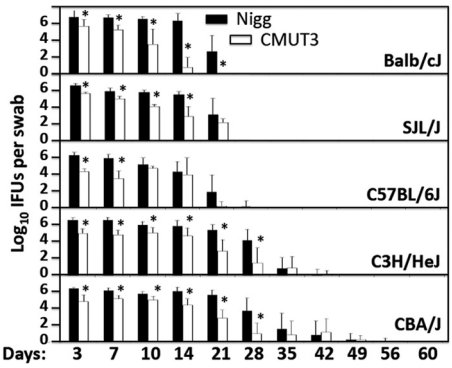

FIG 4 Both *C. muridarum* plasmid-competent Nigg and plasmid-free CMUT3 productively infect the mouse genital tract. The 5 strains of mice intravaginally infected with either *C. muridarum* Nigg or CMUT3 organisms as described in the legend to Fig. 2 were monitored for live organism shedding from the lower genital tract. On different days after infection, as indicated on the *x* axis, vaginal swabs were taken for titration of live organisms in IFU. The $\log_{10}$ IFU were used to calculate the mean and SD for each mouse group at each time point, as shown on the *y* axis. Note that mice infected with plasmid-free *C. muridarum* CMUT3 displayed significantly fewer IFU than plasmid-competent Nigg organisms at most time points, although there was no significant difference in the duration of infection between plasmid-competent and plasmid-free *C. muridarum*. *, significant difference ($P < 0.05$; Kruskal-Wallis).

muridarum can induce hydrosalpinx and infertility in mice (6, 19). The current study has demonstrated that despite the differences in background genes and H-2 haplotypes, all 5 strains developed hydrosalpinx in response to infection with plasmid-competent *C. muridarum*. Importantly, a similar infection with plasmid-free *C. muridarum* failed to induce any hydrosalpinx in the same 5 strains of mice, which is consistent with a previous report (11, 27). We further found that the lack of hydrosalpinx correlated well with reduced infection levels by plasmid-free *C. muridarum* (Fig. 4). The levels of live organisms recovered from vagina/cervix swabs were always lower (although not always with statistical significance) in mice infected with plasmid-free than in mice infected with plasmid-competent *C. muridarum* regardless of the mouse strains and the time points when the swabs were taken within the first 4 weeks of infection. Nevertheless, the lengths of the shedding time courses were similar in mice infected with plasmid-competent and plasmid-free *C. muridarum*. It is worth noting that different strains of mice varied in their overall shedding times, with the longest shedding exhibited by CBA/J and C3H/HeJ mice. However, SJL mice, with a shedding time course that was 2 to 3 weeks shorter than those of CBA/J and C3H/Hej mice (Fig. 4), developed hydrosalpinx with the highest incidence and severity scores (Fig. 2). Apparently, the duration of live organism shedding from the vagina/cervix is less relevant to hydrosalpinx development than the level of shedding. The question is whether the level or the duration of live organism shedding in the lower genital tract can more accurately reflect the live organism infection in the upper genital tract.

Recovery of live organisms from the upper genital tract is significantly reduced in mice intravaginally infected with plasmid-free *C. muridarum*. When segments of genital tract tissues from infected mice, including the vagina/cervix, uterine/uterine horn, and oviduct/ovary, were homogenized for titrating live organisms (Fig. 5A), we found that the numbers of live organisms recovered from the C3H/HeJ mice infected with plasmid-free *C.*

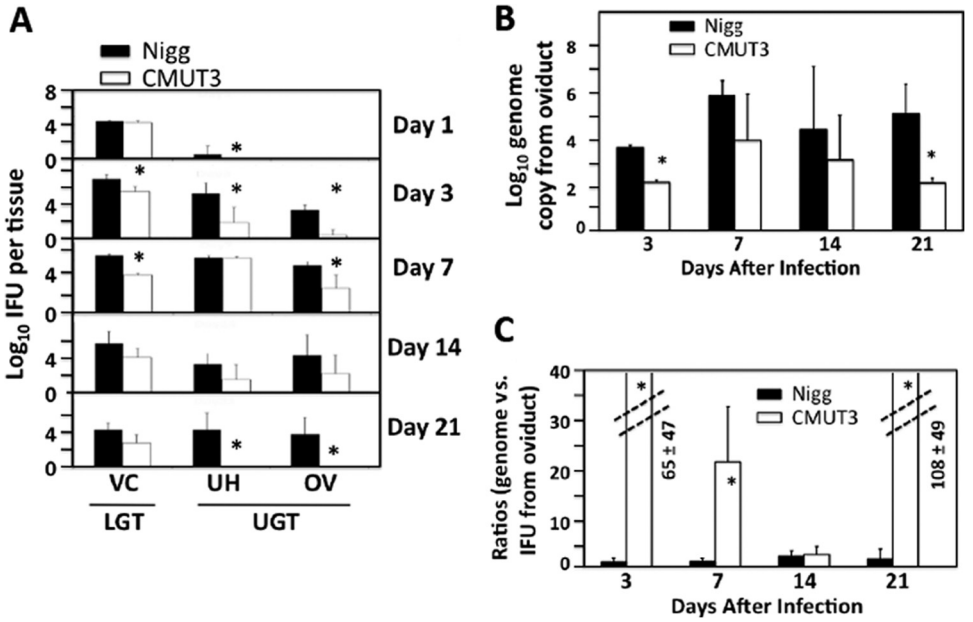

FIG 5 Chlamydial live organisms or genomes recovered from segments of genital tracts of C3H/HeJ mice intravaginally infected with Nigg or CMUT3. C3H/HeJ mice intravaginally infected with *C. muridarum* plasmid-competent Nigg or plasmid-free CMUT3 were sacrificed on different days after infection, as indicated on the right (A) or on the *x* axis (B and C). (A) Genital tract tissues harvested from each mouse were divided into three sections, including the lower genital tract (LGT) vagina/cervix (VC) and the upper genital tract (UGT) uterus/uterine horn (UH) and oviduct/ovary (OV), as indicated. Homogenates prepared from these tissue sections were titrated for infectious organisms, expressed as IFU, which were converted into $\log_{10}$ for calculating the mean and SD for each group at each time point, as shown on the *y* axis. Note that the load of CMUT3 recovered from the oviduct was obviously lower than that of Nigg, and the CMUT3 organisms were not or were only minimally detected on either day 3 or day 21 after infection. $n = 4$ or 5 for each group from 2 separate experiments. (B) *C. muridarum* genome copies in the oviduct/ovary homogenates of C3H/HeJ mice were quantitated using real-time PCR. (C) Ratios of genome copies and infectious organisms (IFU) were calculated for each sample, and the mean and standard deviation from each group are shown on the *y* axis. Note the significantly reduced recovery of live organisms from oviducts of mice infected with plasmid-free *C. muridarum* CMUT3 on days 3, 7, and 21 postinfection. *, significant difference ($P < 0.05$; Kruskal-Wallis).

muridarum CMUT3 were always lower, though not always significantly, than those from mice infected with plasmid-competent *C. muridarum* Nigg. This was especially true 3 days after infection and thereafter. The only exception was the uterus/uterine horn sample harvested on day 7 after infection. Indeed, 7 days after infection was the peak time for plasmid-free *C. muridarum* to reach the upper genital tract tissues, including uterine and oviduct tissues. However, by days 14 and 21, the number of live plasmid-free *C. muridarum* organisms was significantly reduced. Although the recovery of both plasmid-competent and plasmid-free *C. muridarum* organisms from the upper genital tract was reduced by 2 weeks after infection, the reduction in plasmid-free *C. muridarum* organisms was more progressive and accelerated. Since hydrosalpinx is restricted to the oviduct, we further used real-time PCR to quantitate the plasmid-competent and -free *C. muridarum* organisms in the oviduct homogenates (Fig. 5B). Consistent with the live organism recovery, the number of plasmid-free *C. muridarum* CMUT3 genomes was always lower (though not always significantly) than that of plasmid-competent *C. muridarum* Nigg genomes. More interestingly, the ratios of genome copies versus live organisms (IFU) recovered from oviduct samples were significantly higher for plasmid-free CMUT3 than for plasmid-competent *C. muridarum* Nigg (Fig. 5C). This analysis suggests that the viability of plasmid-free *C. muridarum* was significantly lower than that of plasmid-competent *C. muridarum* in the oviduct. The exception was that on day 14 after infection, the genome copy/IFU ratios for both plasmid-competent and -free *C. muridarum* were similar, at ~3, suggesting that one-third of both types of organisms were viable in the oviduct at this time point. However, by day 21, more than 99% of the plasmid-free *C. muridarum* organisms were no longer viable while more than 30% of the plasmid-competent *C. muridarum* organisms remained viable. The rapid loss of viability by the plasmid-free *C. muridarum* organisms may contribute to their inability to induce hydrosalpinx. We further monitored the live organism recovery from genital tract tissues following *C. muridarum* infection in both SJL/J and C57BL/6J mice (Fig. 6), which has allowed us to validate the correlation between oviduct live infection and hydrosalpinx. The numbers of live organisms recovered from the oviducts of mice infected with the plasmid-free CMUT3 were always significantly lower than those from the plasmid-competent Nigg-infected mice. Thus, we have demonstrated a strong correlation between chlamydial oviduct infection and hydrosalpinx induction, which seemed to contradict the claim made in a previous report that the absence of pathology associated with infection by the plasmid-deficient strains was not caused by an inability to establish infection at this site or by a significant decrease in the infectious burden (11, 27).

Plasmid-free *C. muridarum* organisms fail to induce hydrosalpinx and quickly lose viability when directly inoculated into mouse oviducts. The above-mentioned results have allowed us to correlate the reduced survival of plasmid-free *C. muridarum* organisms in the oviduct with the lack of hydrosalpinx following intravaginal infection. To test whether delivery of the same amounts of plasmid-competent and -free *C. muridarum* organ-

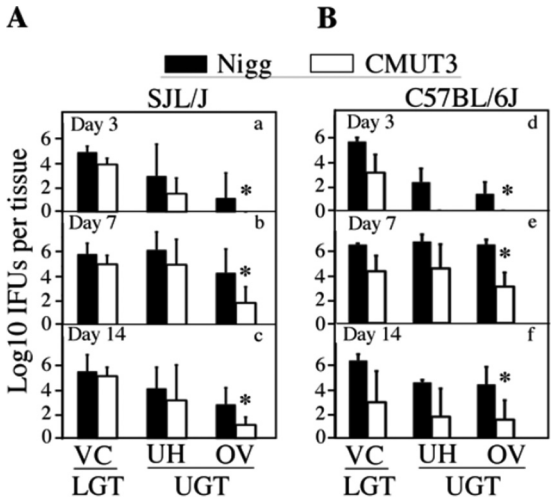

FIG 6 Live organism recovery from segments of the mouse genital tract following intravaginal infection with plasmid-competent or plasmid-free *C. muridarum*. Both SJL/J (A) and C57BL/6J (B) mice intravaginally infected with *C. muridarum* plasmid-competent Nigg or plasmid-free CMUT3 were sacrificed on different days after infection, as indicated in the corresponding graphs. Genital tract tissues harvested from each mouse were divided into three sections as described in the legend to Fig. 5A and displayed on the *x* axes. Homogenates prepared from these tissue segments were titrated for IFU, which were converted into $\log_{10}$ for calculating the mean and SD for each group at each time point, as shown on the *y* axis. Note that the numbers of IFU recovered from CMUT3-infected mouse oviducts/ovaries (OV) were significantly lower than those from Nigg-infected mice regardless of the mouse strain and number of days after infection. *, $P < 0.05$; $n = 5$ for each group.

isms into the oviduct can result in hydrosalpinx by both types of organisms, we used an intrabursal inoculation for the oviduct infection, since intrabursal infection with plasmid-competent *C. muridarum* has been shown to induce infertility in mice (28). As shown in Fig. 7, to our surprise, we found that although plasmid-

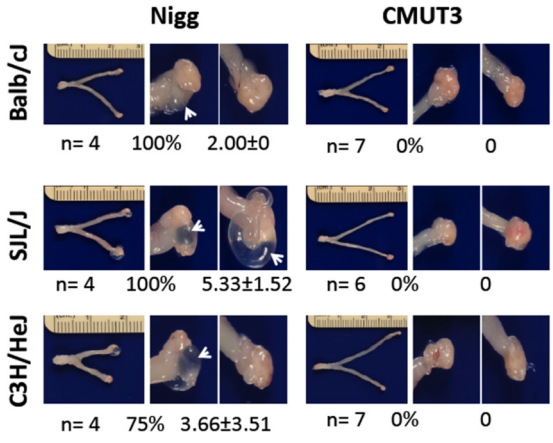

FIG 7 Direct injection of plasmid-free *C. muridarum* into the oviduct fails to induce hydrosalpinx. BALB/cJ, SJL/J, and C3H/HeJ mice intrabursally inoculated with *C. muridarum* plasmid-competent Nigg or plasmid-free CMUT3 organisms were sacrificed 60 days after infection to evaluate the gross pathology, as described in the legend to Fig. 2. The mouse numbers and incidence and severity of hydrosalpinx in each group are shown under the corresponding representative image. Note that although plasmid-competent *C. muridarum* induced severe hydrosalpinx, plasmid-free *C. muridarum* failed to induce hydrosalpinx in any mice.

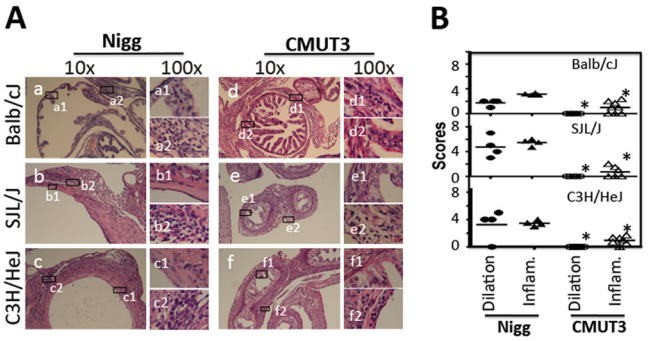

FIG 8 Direct injection of plasmid-free *C. muridarum* into the oviduct fails to induce significant oviduct histopathology 60 days after injection. The oviduct tissues from the experiments described in the legend to Fig. 7 were processed to make sections for H&E staining. The histopathology images (A) were acquired and semiquantitatively scored (B) as described in the legend to Fig. 3. Note that oviducts from all mice infected with Nigg (wild-type *C. muridarum*), but not mice infected with CMUT3 (plasmid-free *C. muridarum*), displayed severe lumenal dilation (filled or open circles) and chronic inflammatory infiltration (Inflam.) (filled or open triangles). *, $P < 0.05$ (Wilcoxon rank sum).

competent *C. muridarum* Nigg induced significant levels of hydrosalpinx in all 3 strains of mice tested, the plasmid-free *C. muridarum* CMUT3 failed to induce any hydrosalpinx in these mice. The hydrosalpinx gross pathology was validated microscopically (Fig. 8). Histopathology revealed that the oviducts of CMUT3-infected mice maintained normal morphology without any detectable lumenal dilation, although some (but significantly reduced) levels of inflammatory infiltration were detected in these oviduct tissues. When the live organisms harvested from vagina/cervix swabs were monitored, although significant levels of plasmid-competent organisms were detected, the plasmid-free *C. muridarum* organisms were barely detectable across the entire infection time course in all 3 strains of mice (Fig. 9). This observation indicated that the plasmid-free *C. muridarum* CMUT3 organisms were not able to survive in the genital tract after intrabursal injection. This assumption was supported by the data shown in Fig. 10, which demonstrated that although similar numbers of Nigg and CMUT3 live organisms and genome copies were detected in the left oviducts of C3H/HeJ mice on day 1 after inoculation into the same side oviduct, the CMUT3 organisms were rapidly reduced by day 7 and completely disappeared by day 14 from the left oviduct tissues. In addition, we also found that although both Nigg and CMUT3 can rapidly descend to the lower genital tract following an intrabursal injection, only Nigg, but not CMUT3, organisms were able to spread to the oviduct tissues on the right side, suggesting that CMUT3 was much less invasive than Nigg in the mouse genital tract.

Induction of hydrosalpinx is dependent on viable *C. muridarum* organisms. The above-described experiments allowed us to correlate a sustained live organism infection in the oviduct with the induction of hydrosalpinx. We next tested whether viable organisms were required for chlamydial induction of hydrosalpinx. In this set of experiments, female SJL/J mice in 4 groups were intrabursally inoculated with 2×10^5 or 1×10^7 IFU of either live or UV-inactivated organisms. Sixty days after inoculation, all mice were sacrificed for examination of genital tract pathologies. We found that live Nigg organisms induced severe hydrosalpinx at both doses (Fig. 11). However, the UV-inactivated Nigg failed to

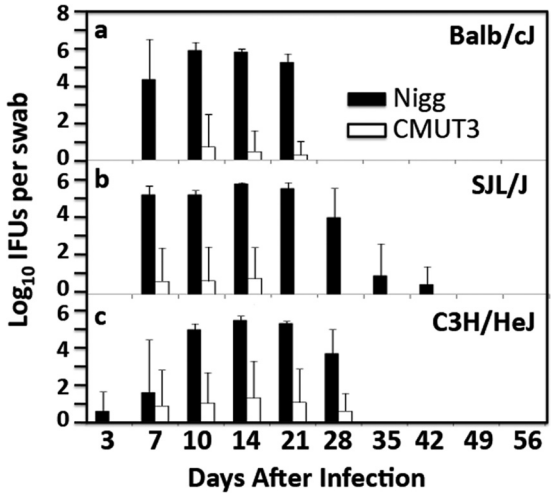

FIG 9 Severely reduced recovery of plasmid-free *C. muridarum* following intrabursal infection. Vaginal swabs were taken from BALB/cJ, SJL/J, and C3H/HeJ mice intrabursally infected with plasmid-competent or plasmid-free (CMUT3) *C. muridarum* on different days after infection, as shown on the *x* axis, for monitoring live organism shedding, expressed as IFU (*y* axis). Note that a significant number of live organisms were recovered from vaginal swabs of mice inoculated with Nigg but only a minimal number of live organisms were recovered from CMUT3-inoculated mice. The error bars indicate standard deviations.

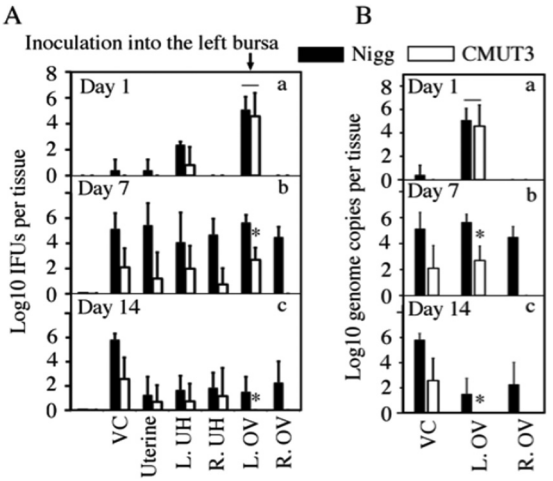

FIG 10 Recovery of chlamydial live organisms or genomes from segments of the genital tracts of C3H/HeJ mice following intrabursal infection with *C. muridarum*. C3H/HeJ mice intrabursally infected with plasmid-competent (Nigg) or plasmid-free (CMUT3) *C. muridarum* were sacrificed on different days after infection, as indicated. (A) Genital tract tissues harvested from each mouse were divided into 6 sections, including the lower genital tract vagina/cervix (VC) and the upper genital tract uterus, left uterine horn (L. UH), right uterine horn (R. UH), left oviduct/ovary (L. OV), and right oviduct/ovary (R. OV), as indicated on the *x* axis. Homogenates prepared from these tissue segments were titrated for infectious organisms, expressed as IFU, which were converted into $\log_{10}$ for calculating the mean and SD for each group at each time point (*y* axis). Note that the IFU of plasmid-free and plasmid-competent *C. muridarum* from the left oviduct/ovary tissues (where the intrabursal injection was introduced) were similar on day 1 but that the plasmid-free *C. muridarum* organisms rapidly disappeared from the oviduct. $n = 5$ for each group. (B) *C. muridarum* genome copies from VC and left and right oviduct/ovary homogenates were quantitated using real-time PCR. *, significant differences ($P < 0.05$; Kruskal-Wallis).

induce any hydrosalpinx even at the inoculation dose of 1×10^7 IFU. Data from histopathology analyses of the same oviduct tissues confirmed that only live Nigg organisms were able to induce lumenal dilation and significant chronic inflammatory infiltration (Fig. 12). When we monitored the recovery of both chlamydial live organisms and genome copies in the vaginal swabs from the above-described 4 groups of mice, we found that, although high numbers of IFU and genome copies were recovered from live Nigg-infected mice, no live organisms and only minimal numbers of genome copies were recovered from the UV-inactivated Nigg-inoculated mice (Fig. 13). Nevertheless, the number of genome copies recovered from the swabs of mice inoculated with 1×10^7 IFU of dead Nigg organisms was similar to that from mice infected with 2×10^5 IFU of live Nigg organisms on day 3 after infection. However, only live Nigg-infected mice developed hydrosalpinx (Fig. 11 and 12). Thus, despite the significant presence of dead Nigg organisms in the mouse genital tract, the dead organisms lacked the ability to trigger hydrosalpinx-causing inflammation.

DISCUSSION

C. trachomatis clinical isolates contain a highly conserved plasmid (29, 30) that has been shown to play a significant role in pathogenesis. The plasmid-free *C. trachomatis* serovar A organisms no longer caused pathology in primate ocular tissues (10), while the plasmid-free *C. muridarum* organisms failed to induce hydrosalpinx (11). However, it is not known whether the highly attenuated pathogenicity of the plasmid-free chlamydial organisms is due to insufficient infection or inability to trigger pathological responses. Here, we present evidence demonstrating that, compared to the plasmid-competent *C. muridarum*, plasmid-free *C. muridarum* organisms cannot establish a robust and sustainable infection in the mouse oviduct, which may contribute significantly to the attenuated pathogenicity. First, plasmid-free *C. muridarum* failed to induce hydrosalpinx in any of the 5 strains of mice that developed severe hydrosalpinx in response to plasmid-competent *C. muridarum* infection. The lack of hydrosalpinx correlated well with decreased levels of plasmid-free live *C. muridarum* organism recovery from the lower genital tract. Second, when segments of genital tract tissues were carefully examined, the plasmid-free organisms not only displayed reduced levels of live organism recov-

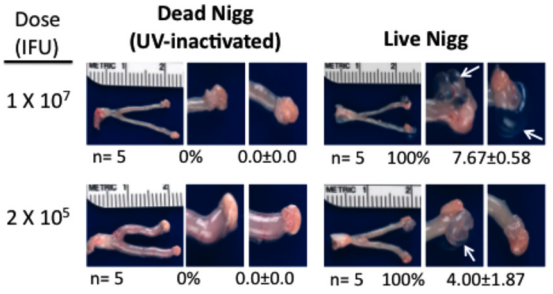

FIG 11 Direct injection of UV-inactivated plasmid-competent *C. muridarum* into the oviduct fails to induce hydrosalpinx. SJL/J mice were intrabursally inoculated with plasmid-competent *C. muridarum* Nigg organisms with or without UV inactivation, and 60 days after inoculation, the mice were sacrificed to evaluate the gross pathology, as described in the legend to Fig. 2. The mouse numbers and incidence and severity of hydrosalpinx in each group are shown under the corresponding representative images. Note that although live *C. muridarum* induced severe hydrosalpinx at either 2×10^5 or 1×10^7 IFU, UV-inactivated *C. muridarum* failed to do so even at 1×10^7 IFU.

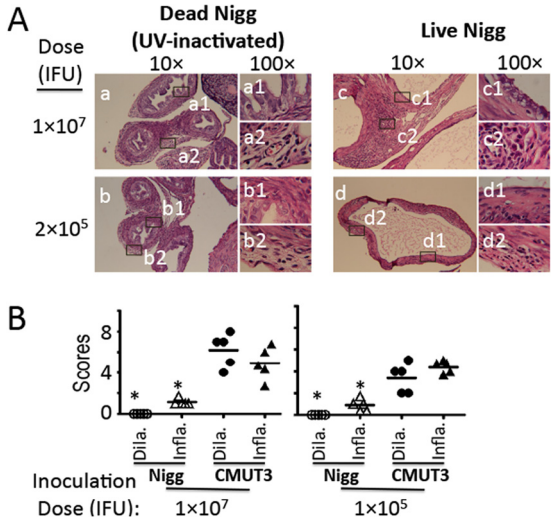

FIG 12 Intrabursal inoculation with dead *C. muridarum* fails to induce oviduct histopathology. The oviduct tissues from the experiments described in the legend to Fig. 11 were processed to make sections for H&E staining. The histopathology images (A) were acquired and semiquantitatively scored (B) as described in the legend to Fig. 3. Note that oviducts from mice inoculated with live, but not mice inoculated with dead, Nigg displayed severe lumenal dilation (filled or open circles) and chronic inflammatory infiltration (filled or open triangles). *, $P < 0.05$ (Wilcoxon rank sum).

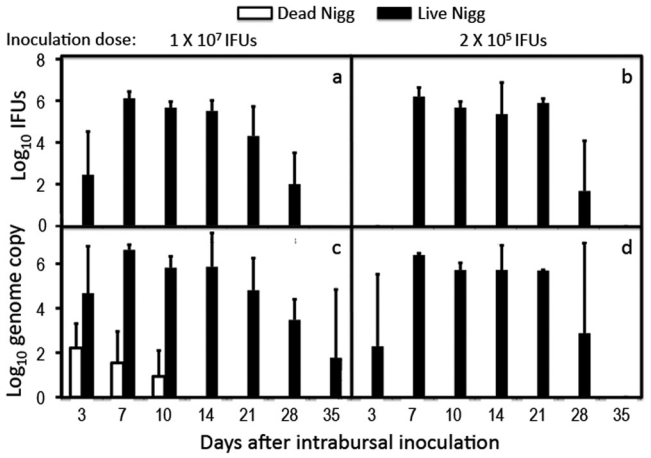

FIG 13 Recovery of live organisms and genomes from vaginal swabs of SJL/J mice intrabursally inoculated with live or UV-inactivated *C. muridarum*. Vaginal swabs were taken from SJL/J mice intrabursally inoculated with live or UV-inactivated (dead) Nigg *C. muridarum* on different days after inoculation, as shown on the *x* axis, for monitoring live organism shedding, expressed as $\log_{10}$ IFU (graphs a and b, *y* axis) or for the number of genomes, expressed as $\log_{10}$ genome copies (graphs c and d, *y* axis). Note that although high numbers of both IFU and genome copies were recovered from live *C. muridarum*-infected mice, no live organisms and only minimal numbers of genome copies were recovered from the dead-organism-inoculated mice. The error bars indicate standard deviations.

ery, but also exhibited shortened infection time courses, especially, in the oviduct. Third, the ratios of genome copy versus infectious organisms recovered from the oviduct were significantly higher for plasmid-free than for plasmid-competent *C. muridarum*, suggesting that plasmid-free *C. muridarum* was less able to survive in the upper genital tract tissues. Fourth, after intrabursal injection, plasmid-free *C. muridarum* organisms were unable to survive in the oviduct for a sustained period of time and were rapidly cleared. Finally, plasmid-competent *C. muridarum* organisms after UV inactivation failed to induce hydrosalpinx when directly inoculated into the oviduct at a dose that was 50-fold higher than the live infection dose. Together, these observations demonstrated that reduced survival and shortened infection with plasmid-free *C. muridarum* organisms may contribute significantly to their attenuated pathogenicity.

The next questions are why and how the plasmid-free organisms failed to cause sustained oviduct infection. There may be multiple mechanisms. First, although we inoculated the same number of IFU of both plasmid-competent and -free *C. muridarum* organisms into the mouse vagina, the plasmid-free organisms may be less infective in the lower genital tract. The limited infection in the lower genital tract may result in reduced ascending and oviduct infection. Indeed, Carlson et al. found an increase of 400-fold in the 50% infective dose (ID_{50}) for plasmid-free lymphogranuloma venereum serovar 2 (L2) organisms based on the live organism shedding from the mouse lower genital tract following intravaginal infection (12), suggesting that plasmid-free L2 organisms are 400-fold less infective in the mouse lower genital tract. Only by infecting mice with the same numbers of ID_{50} of plasmid-competent and plasmid-free organisms may we be able to eliminate the contribution of the differences in lower genital tract infectivity to chlamydial pathogenicity. Second, even when the same ID_{50} is used to infect mice, the difference in chlamydial ability to overcome the mouse cervical barriers responsible for controlling ascending infection may be the next variable that can contribute to chlamydial pathogenicity. The plasmid-free *C. muridarum* organisms may be less capable of ascending to the upper genital tract. Finally, even after excluding the contribution of ascending infection by delivering chlamydial organisms directly into the oviduct via intrabursal injection, the plasmid-free *C. muridarum* organisms still failed to induce hydrosalpinx, which correlated with reduced infectivity in the oviduct. Thus, it will be interesting to test whether the plasmid-free *C. muridarum* organisms are less able to infect oviduct epithelial cells or more susceptible to host defense mechanisms in the oviduct.

The lack of activation of the TLR2 signaling pathway was correlated with a failure to induce oviduct inflammatory pathology following plasmid-free *C. muridarum* infection (11, 27). However, it is not clear whether lack of TLR2 signaling pathway activation during plasmid-free *C. muridarum* infection was due to insufficient infection or lack of ligands (or virulence factors) required for TLR2 activation. In the current study, we found that plasmid-free *C. muridarum* organisms failed to effectively ascend to the oviduct after intravaginal infection compared to plasmid-competent *C. muridarum* organisms. The plasmid-free *C. muridarum* organisms that ultimately reached the upper genital tract were mostly dead by day 21 after infection, while significant numbers of plasmid-competent *C. muridarum* organisms remained viable. The vast difference in viability between plasmid-free and plasmid-competent *C. muridarum* organisms in the oviduct was also observed in the intrabursal infection model. Although the same amounts of live organisms were directly inoculated into the oviduct, significantly fewer plasmid-free *C. muridarum* organisms than plasmid-competent *C. muridarum* organisms were recovered. The rapid loss of viability of the plasmid-free *C. muridarum* organisms in the oviduct may contribute significantly to their fail-

ure to induce hydrosalpinx. This conclusion is further supported by the observation that direct delivery of high-dose UV-inactivated plasmid-competent Nigg organisms still failed to induce hydrosalpinx in the oviduct, suggesting that the viability of chlamydial organisms in the oviduct is required for chlamydial pathogenicity.

Although live organism infection in the oviduct was correlated with the attenuated pathogenicity of plasmid-free *C. muridarum*, it is still unknown how infection with plasmid-competent *C. muridarum* induces hydrosalpinx. Although a TLR2-mediated signaling pathway has been proposed to play a critical role in chlamydial plasmid-dependent pathogenicity (7, 11, 27), it remains unclear whether the TLR2 pathway is sufficient to mediate the hydrosalpinx-causing inflammatory responses. First, the role of the TLR2 signaling pathway in chlamydial pathogenesis was initially proposed based on the observation that TLR2-null ($TLR2^{-/-}$) mice developed significantly reduced inflammatory scores in the mesosalpingeal tissues compared to their heterozygous ($TLR2^{+/-}$) littermates (7). However, severe chronic inflammatory pathology in the oviduct was detected in both $TLR2^{-/-}$ and $TLR2^{+/-}$ mice, with a median oviduct dilation score of ~2 for $TLR2^{-/-}$ and ~3 for $TLR2^{+/-}$ mice (7). Clearly, more careful experiments are required to validate whether TLR2 is required for chlamydial induction of pathology in the oviduct. Second, it is worth noting that the above-mentioned observations were made under a microscope on day 35 after infection, when most inflammatory responses were still reversible. The day 35 inflammatory responses should not be used to represent the irreversible hydrosalpinx visually observed on day 60 after infection. These day 35 inflammatory pathological responses do not necessarily lead to irreversible hydrosalpinx at later times. Clinically, hydrosalpinx refers to solution accumulation in the tube as a result of fibrotic blockage, which is observed in women under a laparoscope. Infertile women laparoscopically diagnosed with hydrosalpinx are usually recommended to accept *in vitro* fertilization for treating infertility after surgical correction of the hydrosalpinges (31). Clearly, hydrosalpinx is considered an irreversible tubal pathology at the clinics. Therefore, the visually detectable hydrosalpinx in the mouse oviduct 60 days after infection is more medically relevant than the microscopically observed inflammation, lumen dilation, and early fibrosis that are still reversible. Thus, more careful experiments are required for testing whether TLR2 is required for chlamydial induction of the long-lasting hydrosalpinx observed under the naked eye on day 60 or later after infection. Third, the observation that TLR2 was activated by infection with plasmid-competent, but not plasmid-free, *C. muridarum* (11, 27) does not necessarily mean that the TLR2 signaling pathway is required for chlamydial pathogenicity and lack of TLR2 activation is the mechanism by which plasmid-free organisms fail to induce pathology. More careful experiments and direct evidence are required to demonstrate a causal relationship between TLR2 signaling and pathogenicity by plasmid-competent or lack of pathogenicity by plasmid-free *C. muridarum*. Finally, and most importantly, the role of TLR2 activation in chlamydial pathogenicity was already disputed by the finding that mice deficient in MyD88, an essential adaptor required for TLR2 signaling, developed more severe hydrosalpinx (observed on days 60 to 80 after infection) with more extensive oviduct infection and Th2-dominant responses (20). These findings were validated by a recent report that further demonstrated a role of MyD88 expressed by $CD4^+$ T cells in clearing oviduct infection (32). Thus, the role of TLR2 in chlamydial pathogenesis deserves careful reevaluation, and more experiments should be encouraged to thoroughly reevaluate the roles of pattern recognition receptors and other inflammatory pathways in chlamydial pathogenesis. The availability of a chlamydial plasmid-based shuttle vector transformation system (33–35) will facilitate the investigation of chlamydial plasmid-dependent pathogenic mechanisms. The plasmid not only includes 8 putative ORFs, but also regulates more than 20 other genes transcriptionally (12). Plasmid-free chlamydial organisms displayed reduced expression of glycogen synthesis genes and lacked glycogen accumulation in the inclusions. Systemically mapping the roles of plasmid-encoded/regulated factors in chlamydial pathogenesis may promote the identification of both chlamydial virulence factors and host inflammatory pathways involved in chlamydial induction of hydrosalpinx.

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